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Extraction and purification of biosurfactant by locally isolated *Enterococcus faecium* bacteria and study of its antimicrobial activity

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In this research, the biosurfactant production by *Enterococcus faecium* isolated from Raw cow's milk was studied when growing in the MRS medium containing Lactose as a carbon source without Tween-80. *E.faecium* supernatant exhibited emulsion action of 60% towards kerosene oil as oil in water, while the surface activity was 34 mm. Biosurfactants with surface tension activity properties were extracted and partially purified from *E. faecium* by cold acetone 3:1, The chemistry structure of the biosurfactant was analyzed, which indicated that it is a glycol-lipo-peptide. Biosurfactant that purified from *E. faecium* showed antibacterial activity by reducing growth of several pathogenic G-ve and G+ve bacteria, including *aureus*, *Staphylococcus epidermidis*, *Streptococcus sp.*, *Staphylococcus Escherichia coli*, *Klebsiella sp.*, *Pseudomonas aeroginosa*, *Acinetobacter* sp. and *Serratia* sp. The inhibitory activity of biosurfactant against pathogenic bacteria was studied by using agar diffusion method, where the biosurfactant had higher inhibitor activity against *E. coli*, where highest diameter of inhibition zone was (23mm). At all concentrations examined, a biosurfactant derived from *E. faecium* considerably decreased bacterial adhesion, albeit to a lower amount.

Keyword: Enterococcus faecium**,** LAB, Biosurfactant, Antibacterial and Antiadhesive activity.

INTRODUCTION

Biosurfactants are hydrophobic and hydrophilic amphiphilic compounds constructed by a category of microorganisms, including bacteria, fungus, and yeast. These molecules display reduced surface tension or interface tension as well as emulsification properties. They are either synthesized extracellularly or bound to cell surfaces (Eras-Munoz *et al*., 2022). In contrast to chemical surfactants, biosurfactants have many benefits, including broad chemical variety, low toxicity, strong biocompatibility, high selectivity, good environmental compatibility, and high biodegradability. (Inamuddin *et al*., 2019; Rodrigues 2015). The advantage of biosurfactant is that they can act in conditions of high pH, temperature, and salt content. It can be produced on several types according to the microorganism produced, such as: lipopeptides, Glycolipid and Glycolipoprotien are showed encouraging antibacterial, antifungal, antiviral, and antiadhesive properties (Moldes *et al*.,2021). Which they can be used instead of traditional antibiotics to combat different food-borne pathogens. There have been reports of different kinds of biosurfactants being produced by bacterial genera including, *Pseudomonas*, Bacillus, Acinetobacter, Halomonas, Arthrobacter, *Lactobacillus* and *Enterococcus* (Giri *et al*.,2019). The goal of this study was to explore the antibacterial and anti-adhesive characteristics of biosurfactant supplied By *Enterococcus faecium* against several pathogenic bacteria Collected from the educational laboratories of the Department of Life Sciences at Al-Mustansiriyah University.

MATERAILS AND METHODS

Microorganisms: *Enterococcus faecium* was isolated from Row cow's milk samples taken from Al-Fadilia cow farm., Baghdad Iraq, samples were transferred to the lab at 4°C. An aliquot (1 gm) was homogenized in 9 mL of sterile 0.9% (w/v) NaCl, diluted 10-fold, and plated on Man Rogosa Sharp agar (MRS) After development the inoculum from the culture flasks was subcultured on bile Esculin agar as confirmative of *Enterococcus spp*. then subcultured on the MRS agar to obtain pure colonies. These isolates were stored at -20°C in MRS broth containing 20% glycerol stock till they were used in this study. Then identified throughout cultural, microscopical, biochemical test and Vitek-2 according to

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(Teixeira *et al*., 2015). Biosurfactant produced from *Enterococcus faecium* was subjected to the test against eight isolates, including *Staphylococcus epidermidis, streptococcus pyogenes, Staphylococcus aureus,Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumanni*, and *Serratia marcescens*. These isolates were obtained from various clinical samples of Iraqi patient, subsequently identified by cultural, microscopic, and biochemical tests in accordance with the guidelines laid out by Forbes (2002) and confirm it with Vitek-2.

***Cultivation of E. faecium and biosurfactant Production*:** The MRS broth utilized for Biosurfactant producing *E.faecium* isolation contains (20 g D-Lactose l^{-}, 10 g peptone 1^{-1}, 10 g Beef extract 1^{-1}, 5 g Yeast extract 1^{-1}, 2g Di-potassium phosphate 1^{-1}, 2g Tri-ammonium hydrogen citrate 1^{-1}, 5g sodium acetate 1^{-1}, 0.2g Magnesium sulphate1^{-1} and Manganese sulfate 1^{-1} without Tween-80. For biosurfactant production, 10 ml MRS broth was inoculated with a fresh culture of *E faecium* and developed at 37°C for 48hrs.and further used as the seed culture for production of biosurfactant. (Salman and Alimer 2014).

***screening for glycolipoprotein biosurfactant production Oil spreading method*:** In order to perform the oil-spreading test, water and oil were placed in a petri dish. A drop of supernatant and put on the oil's surface. For each isolate, the diameter of the obvious zone on the oil surface was measured three times. PBS was utilized as a negative controller (Morikawa *et al*. 2000).

***Determination of emulsification Index (E24%)*:** The Emulsification indicator test was done by following to Sharma *et al*. (2014) approach for assessing biosurfactant activity: The capacity of isolates to emulsify was evaluated using an emulsification index (E24) intended for kerosene oil. In a test tube, 5 mL of Kerosene was mixed with 5 mL of supernatant. The test tube was then rapidly vortexed for two minutes before being let to stand for 24 hours. The ratio of the emulsification index was anticipated using the equation which showed encouraging properties of antibacterial, antifungal, antiviral, and anti-adhesive.

$$E24\% = \frac{\text{Height of the emulsion layer}}{\text{Total height of the mixture}} \times 100$$

***Biosurfactant production, extraction, and partial purification*:** For crude biosurfactant production in flask condition, a pre-culture of 2% (v/v) *E. faecium* was added to 1200 ml of MRS-Lactose (glucose was substituted via lactose) potage (pH 7) and cultivated for 48 hours at 37°C. Cells were separated by centrifugation after 48 hours of incubation (10,000 g, 10 min, 4°C). After cooling centrifuge, the supernatant containing biosurfactant was treated with cold acetone solvent (3:1) (v/v)**,** The mix was surprised continuously at 200 rpm, 4 °C for 15-20 hours inside an incubator shaker, and then centrifuged at 10000 rpm for 15 minutes at 4 °C. The supernatant was discarded, the precipitate was collected and This served as partially purified biosurfactant. The pellet was dissolved in 5 ml D.W., and pH was regulated at 7 with NaOH (1N) as prescribed by (Majid Hussein *et al*, 2015).

***Estimation of Biomass*:** After centrifugation in the biosurfactant extraction process, the cell pellet was cleaned, re-suspended in sterilized water, and centrifuged repeatedly. A cell pellet was later dehydrated until it attained a constant weight in an electric oven at 105°C (Schelegueda *et al.*, 2015).

***chemical analysis of biosurfactant*:** Lipid, carbohydrate and protein content were evaluated with Anschau (2017) method, phenol, and sulphuric acid method by (Dubois *et al.*, 1956) and Bradford method (Bradford, 1976), respectively.

Anti-bacterial Assay

***Agar diffusion method*:** Pathogenic bacterial strains *Staphylococcus aureus, Escherichia coli streptococcus pyogenes, Klebsiella Staphylococcus epidermidis, pneumonia, Pseudomonas aeroginosa, Acinetobacter baumanni* and *Serratia marcescens*, were obtained from various clinical samples of Iraqi patients and Muller-Hinton Agar (MHA) prepared plates were swapped with these pathogenic bacteria. Wells were made in the agar plates using a sterile well maker and then 100 µl of a biosurfactant with a 40 mg/ml concentration were added to a separate well. The plates were incubated for 24 hours at 37°C. The diameter of the clear zone was observed (Giri *et al.*, 2019 and Hussein and Hasan., 2022).

***Anti adhesion Assay*:** According to the method established by Ali (2012), co-incubation studies were used to measure the anti-adhesion activity of the partially purified biosurfactant obtained from *E. faecium* against Staphylococcus epidermidis, Staphylococcus aureus, and streptococcus pyogenes *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeroginosa, Acinetobacter baumanni* and *Serratia marcescens*. The inhibition of adhesion ratios of partial purified biosurfactant for each pathogenic bacteria was determined as equation termed by Gudina *et al.* (2010).

$$\text{Inhibition of Adhesion \%} = [1- (\frac{A}{A0})] \times 100$$

A: represent the absorbance of the well with a biosurfactant and A0: the absorbance of the control well.

RESULTS AND DISCUSSION

***Initial isolation*:** The bacterial isolate was examined based on its morphological and biochemical characteristics, All isolates grew on bile esculin agar. This media a choosy differential agar used to isolate and identify members of the genus *Enterococcus*. Esculin is the differentiating element, whereas bile salts are the selective ingredient. Esculin is hydrolyzed by Enterococcus into combined products with ferric citrate in the medium to form insoluble iron ions, Black hallow colonies on Bile Esculin agar was considered as presumptive enterococci colonies (Davis *et al*., 2022). Enterococci were observed small, smooth, entire margin and white colonies on

MRS agar (Ben Braiek *et al*.,2017). and Identification was performed based on the Vitek2 system (Forbes *et al*.,2007; Sarwar *et al*.,2021).

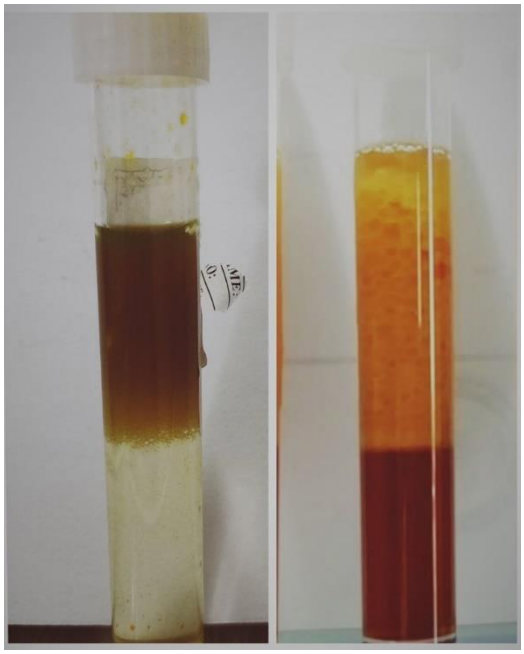
***Screening for biosurfactant production*:** In the current investigation, *E. faecium* isolates were screened for biosurfactant production by two methods: Oil spreading method and Emulsification Index 24%

***Oil Spreading test*:** The tested *Enterococcus faecium* isolate showed the highest oil spreading activity with diameter 34 mm. Results are given in Fig. 1.

The results indicated the *E. faecium* had the ability to synthesize biosurfactants. The diameter of the clearance zone formed by biosurfactant-comprising solution has been shown to be directly proportional to the concentration of biosurfactant (Youssef *et al*., 2005), Ghazi Faisal *et al.* (2023) reported that this method is better than other screening methods due to be rapid and easy to carry out, requires no specialized equipment. while Lamia *et al.* (2021) indicated that the development of clear zone on surface of oil is a unique quality of surfactants, which, together with tensile surface, provides an index to estimate the performance of biosurfactant-making bacteria .

Figure 1. The spreading of partial biosurfactant produced by *E. faecium* on oil surface.

***Emulsification activity E24%*:** Emulsification index (E24%) of *E. faecium* was determind with bacterial supernatant and kerosene oil. *E. faecium* showed good emulsifying activity, emulsion formed with E24 (60%), Figure 2 shows the E24% result. According to Das *et al*. (2009) the emulsification index corresponds to the surfactant concentration. For another studies Lactic acid bacteria formed good emulsifying ability (Brzozowski *et al.,* 2011), While Ghasemi *et al* (2019) reported that the Biosurfactant produced by lactic acid bacterium *Pediococcus dextrinicus* SHU1593 is able to emulsify n-hexadecane (58%) and kerosene (56%).



A B

Figure 2. The Emulsion form of *E. faecium*
A: phosphate buffer saline, B: *E. faecium* supernatant

***Extraction and Partial Purification of the Biosurfactants*:** The crude Biosurfactant produced by *E.faecium* was extracted and partially purified by using cold acetone: Supernatant cell culture (3:1) (Al-Rrubaie *et al*.,2019). Arsyah *et al.,* (2018) also selected the cold acetone method for the extraction and purification of biosurfactants. This occurs as a result of the availability of the hydrophobic end of the biosurfactant, which makes them soluble in acetone or organic solvent. (Shah *et al.,* 2016). In current study, the yield of partially purified biosurfactant was (9.4 g/2.1 gm Biomass /1 liter of culture medium) from *E.faecium* in the presence of Lactose as sole source of carbon.

The results of chemical composition analysis, the biosurfactant produced by *Enterococcus faecium* was content of Glycolipoprotein. This study indicates that the lipid content of purified Biosurfactant was 39.7% according to Anschau standard curve. According to Dubois standard curve the carbohydrate contents were 33% and the concentration of the protein was 9.5%, according to an analysis of the protein's absorbance using the Bradford standard curve.

***Antibacterial activity of biosurfactant*:** Glycolipoprotein from *E. faecium*, showed antibacterial activity against tested isolates with different degree (Table 1). From those results, the Biosurfactant gave the largest inhibition zone (23 mm) against *E. coli*. Biosurfactant very efficiently suppressed the growth of tested G-negative and G- positive bacteria. It is significant to mention that the biosurfactant produced by *E. faecium* demonstrated good effect against bacterial infections. Various biosurfactants with antibacterial activity have already

been reported. However, few researchers have investigated the antibacterial action of biosurfactants isolated from *Enterococci*. Similarly, biosurfactant formed from *Lactobacillus rhamnosus* exhibited antimicrobial effect against some bacteria causing UTI in Iraqi women (Salman and Alimer; 2014), *Lactobacillus casei*-derived biosurfactant displayed antimicrobial activities against *S. aureus* strains (Merghni *et al.*, 2015). also, biosurfactant produced from *Bacillus* strain had the highest inhibitory zone against tested E. coli MTCC (13 ± 0.5 mm)(Giri *et al.*,2019), biosurfactant produced by *Pediococcus dextrinicus* had significant a complete antimicrobial effect against *E. coli*, *E. aerogenes*, and *P. aeruginosa* at concentration 25 mg/ml (Ghasemi *et al.*,2019), another studies, biosurfactant produced by Lactic Acid Bacteria appeared antibacterial result against Gram positive and Gram negative bacteria (Hassan, (2018) ; Dong *et al.*, 2019 and Nataraj *et al.*, 2021).

Table 1. Antibacterial effect of biosurfactant produced by *E. faecium*.

Pathogenic bacteria	Inhibition zones(mm) at 25 mg/ml
K. pneumonia	17
S. epidermidis,	20
S. pyogenes,	19
P. aeroginosa	15
E. coli	23
A. baumanni	12
S. marcescens.	16
S. aureus	21

The biosurfactant's mechanism of antibacterial activity focuses on the fact that the structural likeness to detergent provided by the existence of the hydrophilic head and lipophilic tail allows for its penetration into the lipid bilayer of the cell membrane, producing disruption (Malakar, and Deka, 2021), Another reasoning for how biosurfactants work as antimicrobials is the intercalation of biosurfactant into the membrane causes the formation of holes in the cell membrane. Also Nucleic acid leakage among other intercellular materials, other routes for the manifestation of antibacterial activities include interactions with membrane phospholipids or modifications to the electrical conductivity of membranes (Abdelli *et al.*,2019; Sharma and Sahran 2016). The other hand, the anti-adhesive effects of partial purified biosurfactant was assessed against tested pathogenic bacteria (Table 2). The partial purified Biosurfactant showed anti-adhesive activity against all tested bacteria, but anti-adhesive activity was dependent on biosurfactant concentrations the highest anti-adhesive percentage was observed for *E. coli* (78%) at a concentration of biosurfactant 50 mg/ml .

Biosurfactant produced from *E. faecium* against *E. coli* and *S. aureus* was comparable to the results attained with biosurfactant produced by *Lactobacillus brevis* and *Bacillus* sp. which inhibited adhesive of *E. coli* and *S.aureus* (Haddaji *et al.*,2022). Shawkat *et al.* (2019) showed that the biosurfactant even at low to moderate concentrations, it proved efficient against *Salmonella enteritidis*, *Staphylococcus epidermis*, and *E. coli* but, low activity was demonstrated for *Staphylococcus aureus*. According to Rodrigues *et al.* (2006), the primary objective of a biosurfactant is to alter the surface's physicochemical characteristics in order to decrease the attraction that microorganisms have to its surface.

Table 2. Anti-adhesive activity of partial purified biosurfactant produced by *E. faecium*.

Pathogenic bacteria	Anti-adhesive percentage (%) of biosurfactant at mg/ml				
	3.12	**6.25**	**12.5**	**25**	**50**
K. pneumonia	4	5	5	7	10
S. epidermidis,	10	18	27	43	59
S. pyogenes,	12	16	21	39	64
P. aeroginosa	15	20	23	46	67
E. coli	13	19	20	43	78
A. baumanni	19	23	25	38	55
S. marcescens.	2	5	6	10	13
S. aureus	20	28	35	55	72

When compared to the control, positive percentages show a decrease in bacterial adhesion, the percentage 0% was selected as the negative control in order to demonstrate that the biosurfactant isn't present.

Conclusion: In conclusion, It was observed that *E.faecium* have the ability to create biosurfactant with antibacterial and antiadhesive property against some tested pathogenic isolates *Staphylococcus aureus Escherichia coli, streptococcus sp, Staphylococcus epidermidis, Klebsiella sp.,Pseudomonas aeroginosa, Acinetobacter sp. and Serratia sp.*

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